

## Pharmacology of Thrombolytic Drugs

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Streptokinase and urokinase have proved to be useful in a limited number of clinical conditions. Mainly because of the risk and unpredictability of bleeding with this first generation of thrombolytic agents, thrombolysis has not been ingrained in medical practice. In the interim, more fibrin-specific thrombolytic agents have been developed such as acylated streptokinase-human plasminogen complex, tissue-type plasminogen activator (t-PA) and single chain urokinase-type plasminogen activator (scu-PA or pro-urokinase). Only the latter two drugs do not induce major systemic fibrinogenolysis at thrombolytic effective doses. These two agents, obtained

by recombinant techniques, as well as acylated streptokinase-plasminogen complex are available for clinical investigations.

The first results of systemic administration of recombinant tissue-type plasminogen activator (t-PA) in patients with acute myocardial infarction were published and are promising. Continued experimentation with t-PA and pro-urokinase in evolving myocardial infarction and other thrombotic disorders is essential to better delineate their therapeutic index.

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The principal aim of thrombolytic therapy is the removal of a pathologic intraluminal thrombus or embolus that has not been dissolved by spontaneous fibrinolysis. In certain clinical conditions such as acute myocardial infarction, the speed of thrombus dissolution is critical because only early reperfusion can reduce the infarct size and improve jeopardized left ventricular function. The same concern for rapid lysis of emboli prevails in life-threatening, massive pulmonary embolism. Although direct local delivery of thrombolytic agents at the site of the vascular occlusion may enhance its lysis, there is an inherent time lag required for coronary and pulmonary artery catheterization with its unavoidable delay in drug administration (1).

Regardless of whether systemic or regional administration is selected, thrombolytic treatment should be safe. Bleeding complications are the main reason why streptokinase and urokinase have not been ingrained in medical practice. The novel, more fibrin-specific thrombolytic agents tissue-type plasminogen activator (t-PA) and pro-urokinase (now termed single chain urokinase-type plasminogen activator [scu-PA]) are more promising in this regard. The latter drugs are also easier to use because, at effective thrombolytic doses, no or only limited systemic fibrinolytic activation occurs, avoiding the undesirable breakdown of the

hemostatic system and eliminating the need for laboratory monitoring.

### Streptokinase

Streptokinase is a nonenzymatic protein isolated from the broth of Lancefield group C strains of beta-hemolytic streptococci. It is a single polypeptide chain without carbohydrates, with a molecular weight of 47 kilodaltons (2). The amino acid sequence of streptokinase is known and is similar to that of *streptomyces griseus* and human trypsin (3,4).

Streptokinase is an indirect activator of plasminogen. It initially forms a 1:1 stoichiometric complex with the zymogen plasminogen, which thereby undergoes a transition, allowing exposure of the active site within plasminogen without apparent peptide bond cleavage (5-8). This modified streptokinase-plasminogen complex behaves as a plasminogen activator (9). The activator complex can also induce a limited proteolysis in the plasminogen molecule of other streptokinase-plasminogen molecules whereby plasminogen is converted to plasmin; streptokinase progressively degrades to smaller fragments, resulting in a gradual loss of activator activity (10-12).

**Mode of action.** After the injection of indium-131-labeled streptokinase, an immediate rapid clearance phase (half-life 18 minutes) is followed by a slower disappearance phase (half-life 83 minutes) (13). The initial rapid phase is interpreted as immune complexing of streptokinase. Indeed, hu-

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man plasma contains antibodies directed against streptokinase, probably as a result of previous infections with beta-hemolytic streptococci. The titer of streptokinase antibodies varies from person to person (14). Because streptokinase reacts with antibodies and is thereby rendered biochemically inert, sufficient amounts of streptokinase must be infused to neutralize the antibodies before fibrinolytic activation is obtained (15). If a loading dose of streptokinase high enough to overcome the streptococcal antibodies is given, plasminogen will be activated to plasmin. Low doses of streptokinase may generate more plasmin and less streptokinase-plasminogen complex; however, the plasmin formed will soon be neutralized by circulating antiplasmin. Only when the  $\alpha_2$ -antiplasmin level is markedly reduced will plasmin be less rapidly neutralized and exert a proteolytic effect on several plasma proteins, among which the coagulation components fibrinogen and factors V and VIII are the most important.

A high initial dose of streptokinase will be associated with a rapid and profound reduction in the level of circulating plasminogen, antiplasmin and fibrinogen (14). Provided a high maintenance dose of streptokinase follows, high circulating activator levels are obtained and fibrinogen levels start to rise in the subsequent 24 hours (16).

Excessive lowering of the concentrations of these blood coagulation components in plasma, combined with the inhibitory effect of some fibrinogen degradation products on platelet aggregation and fibrinogen polymerization, are considered responsible for the potentially dangerous bleeding tendency (17-19). However, the magnitude of systemic fibrinogenolysis and its laboratory derangements do not strictly correlate with bleeding manifestations (20-22).

### Equimolar Streptokinase-Plasminogen Complex and Its Stable Acyl Derivatives

Equimolar complexes of streptokinase and human plasminogen have been used for thrombolysis because their fibrinolytic activity does not depend on the circulating plasminogen content (23,24) and also because the plasminogen conformation is somewhat changed in the complex, resulting in a better catalytic activity (25).

**Clinical application.** The clinical use of this complex was attempted after a favorable report (26) of the sequential intravenous use of 90-120 mg of human Lys-plasminogen, followed by 600,000 IU of streptokinase. With 100,000 IU/h of equimolar streptokinase-plasminogen complex, administered in a continuous infusion over 2 days, the residual plasminogen remained between 10 to 20% of the normal value while the fibrinogen levels decreased by two-thirds (27). These investigations were conducted in patients with up to 6 week old thrombotic occlusions of the femoropopliteal artery, of which 73% were cleared. However, serious cerebrovascular side effects were encountered.

The hope that the streptokinase-plasminogen complex would not be immunogenic in human patients could not be substantiated (28). Clinical experience with equimolar mixtures of streptokinase and human plasminogen is limited, and streptokinase-plasmin beta-chain complexes are almost nonexistent (29). It should be noted that the lysine binding sites of plasminogen responsible for its binding to fibrin are located in the alpha-chain, theoretically rendering a plasmin beta-chain-streptokinase complex less fibrin-specific.

**Acyl derivatives.** Stable acyl derivatives of an equimolar streptokinase-plasminogen complex have been prepared by acylation of the Ser 740 residue located in the catalytic center of the light beta-chain of plasminogen (30,31). Because the catalytic center is functionally separate from its fibrin-binding site located in the heavy or alpha-chain of plasmin, acylated streptokinase-human plasminogen is catalytically inert so that it can circulate in the vascular system without reaction with either plasma inhibitors or plasminogen, but still bind to fibrin through the unmodified kringle domains of the plasminogen moiety. These compounds deacylate under physiologic conditions after first order kinetics, with half-lives of 40 minutes (p-anisoyl derivative, BRL-26921) and 17 hours (p-aminobenzoyl derivative, BRL-33575).

In a thrombosis model in the rabbit, acylated streptokinase-plasminogen was significantly more thrombolytic than the unmodified streptokinase-plasminogen complex, both given as bolus doses (31). In a jugular vein thrombosis model in dogs, streptokinase-plasminogen results in a marked decrease to 20% of the original fibrinogen level at a dose that is not yet thrombolytic, while the two acylated compounds used at equivalent streptokinase doses were thrombolytic but also markedly reduced the fibrinogen level (32,33). In human volunteers, 5 mg of the BRL-26921 compound had little effect on the systemic fibrinolytic system; on a molar basis, this dose is equivalent to 178,000 IU of streptokinase and does produce a significant decrease in fibrinogen and plasminogen (34). At doses greater than 5 mg, progressive reductions were observed in plasma levels of fibrinogen, plasminogen and  $\alpha$ -antiplasmin (35).

**Clinical application.** The clinical indication for which acylated streptokinase-plasminogen complexes have been best studied is acute myocardial infarction. With an intracoronary bolus injection of 10 mg or more of BRL 26921, angiographically determined reperfusion rates of 75% were obtained in 74 patients (36). After intravenous bolus injection of 30 mg of BRL 26921, coronary reperfusion was achieved in 86% of 57 patients. Several minor bleeding episodes were reported in most cases, and with the latter dose, fibrinogen levels were reduced by as much as 80% of the preinfusion value (37). Other side effects were those usually observed after streptokinase. The main advantage of acylated streptokinase-plasminogen complexes seems to be that they can be given as an intravenous bolus injection.

## Urokinase

The presence of a fibrinolytic activity in urine was discovered in 1913 by Johansson (38). Much later, it was found that the urine activates plasminogen (39); the plasminogen-converting principle was recognized as a kinase, hence the name urokinase (4). Because this term is a misnomer, the Subcommittee on Fibrinolysis (41) recommends the designation two-chain urokinase-type plasminogen activator. Urokinase has been isolated and purified from human urine (42) and later from cultured human embryonic kidney cells (43) and certain tumor cells (44). The gene coding for urokinase has been cloned and expressed in *Escherichia coli* (45).

Urokinase is a trypsin-like serine protease composed of two polypeptide chains (20 and 30 kilodaltons) connected by a single disulfide bridge. Urokinase may occur in two molecular forms: a high molecular weight form (54 kilodaltons) and its proteolytic product or a low molecular weight form (31 kilodaltons) that contains mainly the heavy chain (the high molecular weight form apparently predominates in humans) (46). The complete primary structure of urokinase has been elucidated (47); the heavy and light chains contain, respectively, 253 and 157 amino acids.

**Mode of action.** In contrast to streptokinase, urokinase is a direct activator of plasminogen and cleaves, by first-order kinetics, a single Arg 560-Val 561 bound in the plasminogen molecule (9). Although in vitro high molecular weight urokinase has greater clot-dissolving activity than does the low molecular weight form (48), this difference could not be confirmed in vivo (49). Urokinase prepared from either urine or tissue culture is not antigenic in humans (50), and both have a similar esterase and fibrinolytic activity in vitro and in vivo (49,51).

Urokinase differs from tissue-type plasminogen activator both in its antigenic characteristics and in its enzyme specificity, particularly with respect to the activation of fibrin-associated plasminogen (52,53). The mean clearance half-life of urokinase in humans is  $14 \pm 6$  minutes (54). From subsequent turnover studies of urokinase in rabbits (55,56) and squirrel monkeys (56), it was concluded that urokinase is rapidly removed from the blood by clearance and degradation in the liver. Recognition by the liver does not require a functional active site and is not mediated through carbohydrate side chains. Inactivation by plasma protease inhibitors ( $\alpha_2$ -antiplasmin,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -antitrypsin and antithrombin III) does not seem to play a significant role in the inhibition of urokinase in vivo (57-60). However, urokinase antigenic protein has been detected in human plasma (61,62) and more recently, evidence has been presented for a new fast-acting inhibitor of urokinase (and of t-PA) in very low concentrations in the blood (63).

**Clinical applications.** In most therapeutic trials a loading dose of 2,500 to 4,500 Committee on Thrombolytic

Agents (CTA) units per kg body weight has been administered in a short (16 to 60 minutes) intravenous infusion, followed by the same hourly maintenance dose for 12 to 24 hours. With this dose, a mild depletion of plasminogen and fibrinogen is noted with marked fibrinolytic activity in circulating plasma; higher doses reduce the plasminogen and fibrinogen levels to a similar extent as streptokinase (49,54).

A multicenter study (64) in patients with recent pulmonary embolism has compared a short 12 hour treatment with 4,400 CTA units of urokinase/kg per h and a 24 hour treatment with 2,000 CTA units/kg per h together with heparin. There was no significant difference between the two regimens in the changes seen in the pulmonary angiogram. Bleeding complications were also the same with the two treatments, as was the incidence of recurrent embolism occurring within 24 hours of stopping thrombolytic treatment. A multicenter trial (65) was conducted in France to compare the 12 hour treatment with 4,400 CTA units of urokinase/kg per hour and the 24 hour treatment with half this hourly dose in patients with recent pulmonary embolism. The angiographic results and bleeding complications were not significantly different between the two regimens. However, either treatment tested would be too short to dissolve concomitant deep vein thrombi effectively, which requires 4 to 5 days of intense thrombolysis. The best prevention of recurrent pulmonary embolism is, indeed, complete phlebographic clearance of deep vein thrombosis (66,67).

## Tissue-Type Plasminogen Activator

**Structure.** Tissue-type plasminogen activator (t-PA) has been isolated and purified from human uterine tissue (68) and a human melanoma cell line (Bowes, RPMI-7272) (69); more recently, the gene for human t-PA has been cloned and recombinant t-PA expressed (70). Tissue-type plasminogen activator is a serine protease with a molecular weight of approximately 70 kilodaltons; t-PA obtained from Bowes melanoma cells appears to exist in two variants with an apparent molecular weight difference of 3 kilodaltons (71,72). The concentration of t-PA in human plasma is approximately 5 ng/ml (circa 0.1 nM). Human t-PA consists of one polypeptide chain containing 527 amino acids, exhibiting serine as the  $\text{NH}_2$  terminal amino acid (70). After limited plasmic action, the molecule is converted to a two-chain activator linked by disulfide bonds (69,73,74). This occurs by cleavage of the Arg 275-Ile 276 peptide bond, yielding a heavy chain (36 kilodaltons) derived from the  $\text{NH}_2$  terminal part of the molecule and a light chain (32 kilodaltons) comprising the  $\text{COOH}$  terminal region. The heavy chain contains two regions that share a high degree of homology with the five kringles in plasminogen and with the single kringle in urokinase. The molecule contains an  $\text{NH}_2$  terminal region that is homologous with the finger domains responsible for the fibrin affinity of fibronectin (75) and another sequence hav-

ing homology with human epidermal growth factor. The catalytic site is located in the light or beta-chain of t-PA; it contains the three residues common to all trypsin-like serine proteases (histidine in position 325, aspartic acid in position 374 and serine in position 481) and is highly homologous to corresponding parts of trypsin, thrombin, plasmin and elastase (70,76). The major differences among the mammalian serine proteases occur in exposed areas and external loops (77,78).

**Mechanism of action.** Purified tissue-type plasminogen activator (t-PA) binds specifically to fibrin (79-82) or cyanogen bromide (CNBr)-digested fibrinogen fragments (83). Quantitative data have been obtained on t-PA binding to fibrin and on the role of fibrin in the activation of plasminogen by t-PA (54,84,85). It was shown that t-PA has a weak affinity for plasminogen ( $K_m = 65 \mu M$ ) in the absence of either solid phase fibrin or a CNBr-digested fibrinogen, but a much higher affinity when fibrin is present ( $K_m = 0.16 \mu M$ ). Kinetic data support a mechanism in which t-PA and plasminogen adsorb to a fibrin clot in a sequential and ordered way, yielding a ternary complex (54).

No difference was found in these kinetic variables for either the t-PA of melanoma origin (69) or that obtained by recombinant DNA technology (70). The structures involved in the binding of t-PA to fibrin are not clearly defined. The  $NH_2$  terminal region of t-PA, which is homologous to the finger region of fibronectin, has been implicated (75), but recent observations seem to be at variance with this hypothesis (86). On the fibrin surface, light chain t-PA is quickly converted to a two-chain form during fibrinolysis, but there is no evidence that this conversion plays a role in the regulation of fibrinolysis (87).

This increased affinity appears to be the result of a "surface assembly" of t-PA and plasminogen on the fibrin surface. Fibrin essentially increases the local plasminogen concentration, creating an additional interaction between t-PA and its substrate through a cyclic fibrin bridge and resulting in a low Michaelis-Menten constant for the activation of plasminogen by t-PA (54). Plasmin formed on the fibrin surface has both its lysine-binding sites and active sites occupied and is, thus, only slowly inactivated by  $\alpha_2$ -anti-plasmin (half-life  $\approx 10$  seconds). Liberated plasmin is, however, very quickly inactivated by  $\alpha_2$ -antiplasmin (half life  $\approx 100$  ms). Effective thrombus dissolution in vivo, thus, requires a continuous replacement at the fibrin surface of neutralized plasmin by new plasminogen molecules.

The one-chain and two-chain forms of t-PA have different amidolytic activities toward low molecular weight substrates (88). They have, however, virtually the same fibrinolytic activity in a purified system, and the plasminogen-activating properties are also similar (74,87). Several laboratories have obtained evidence for the existence of a rapid-acting inhibitor (or inhibitors) of t-PA at low concen-

trations in plasma of healthy individuals (89-92) or at higher levels in pathologic plasma samples (93-95). The complex formed between t-PA and its specific inhibitor has a molecular weight of 110 kilodaltons and is formed very rapidly (second order rate constant  $10^7 M^{-1}s^{-1}$  (96).

**Clinical effects.** Pharmacokinetic studies in human volunteers (96a) and patients with myocardial infarction (97) have shown two compartment behavior of t-PA, with a disappearance from plasma with alpha-phase half-life of 5.7 minutes, a half-life of 1.3 hours and a total clearance of 380 ml/min. Experience with t-PA is limited to about 500 patients with acute myocardial infarction (98-103). With the doses used, a decrease in fibrinogen concentration to about 50% of the preinfusion value was noted. It was shown that systemic activation of the fibrinolytic system is dependent on the dose and infusion rate, as could be anticipated from the kinetic variables of the activation of plasminogen by t-PA (105). This fibrinogen decrease might be due, in part, to in vitro fibrinogen degradation. It was noted (103) that the fibrinogen level also decreased in the placebo-treated patients by 11%, indicating that coronary catheterization and hemodilution could also be partly responsible. Both hematoma at the catheter site and prolonged bleeding at puncture sites were half as frequent during t-PA treatment compared with streptokinase treatment in a comparative trial (102). Further experience is required to substantiate whether less bleeding is encountered when using t-PA for therapeutic thrombolysis as compared with streptokinase or urokinase.

It was noted in the two European trials with t-PA (102,103) that the observed increase in fibrinogen degradation products is less than anticipated from the reduction of circulating fibrinogen (104,105). It is possible that the fibrinogenolytic breakdown is limited to the generation of the early degradation products, fragments X or Y, or both, which are slowly coagulable or incorporated in fibrin clots. This hypothesis is further supported by the less substantial decrease in fibrinogen during t-PA treatment when measured by the sodium sulphite precipitation method (106), which assesses both the fibrinogen and fragments X and Y, rather than with a coagulation rate assay (107).

### Single Chain Urokinase-Type Plasminogen Activator (scu-PA) or Pro-Urokinase

Several groups (108-114) have reported the isolation of an inactive single chain precursor of urokinase that, after selective cleavage of the Lys 158-Ile 159 peptide bond by plasmin, is converted to a fully active two-chain structure. This single chain precursor of urokinase is, however, a true enzyme (115).

**Mechanism of action.** The mechanism for the activation of plasminogen by scu-PA has been recently elucidated (116);

scu-PA directly activates plasminogen to plasmin. The generated plasmin then converts scu-PA to urokinase, which in turn activates plasminogen to plasmin. In a plasma milieu, activation of plasminogen by scu-PA is prevented by competitive inhibition.

A detailed kinetic analysis (116) revealed that scu-PA is an equally potent activator of plasminogen as urokinase. This is due to the fact that the proenzyme, which is virtually inactive toward low molecular weight substrates for urokinase, forms an intermediate of the Michaelis-Menten type with plasminogen, with a much higher affinity than that of the active enzyme with its substrate (117).

A urokinase-type plasminogen activator can be purified from conditioned media of several human cell cultures, particularly from the human lung adenocarcinoma line CALU-3 (119). This material is kinetically identical to scu-PA. In human urine, the urokinase-related proteins consist of about 25% of scu-PA (10 to 20  $\mu\text{g/liter}$ ) and of about 75% two-chain urokinase (40 to 50  $\mu\text{g/liter}$ ), the bulk of which is complexed to an inhibitor (118).

The gene coding for urokinase has recently been cloned and expressed in *E. coli* (45). However, if proteolytic degradation is carefully avoided during purification, scu-PA may be obtained from the same expression system (114). Natural and recombinant scu-PA can induce more clot-selective thrombolysis than can natural or recombinant urokinase in experimental thrombotic models (60,111,119,120).

**Thrombolytic effects.** Coronary thrombolysis was obtained after intravenous infusion of scu-PA in dogs (111) and baboons (Flameng et al., unpublished observations, 1986). This thrombolytic effect, obtained after  $23 \pm 2$  minutes in four dogs and  $21 \pm 4$  minutes in six baboons, was not associated with systemic fibrinolytic activation.

Scu-PA was administered to six patients with evolving myocardial infarction of less than 5 hours' duration and angiographically confirmed total occlusion of the infarct-related coronary artery (120a). In four of the six patients, complete reperfusion was obtained during intravenous infusion and in one patient after 20 minutes of intracoronary infusion; one patient did not respond. The infusion caused significant changes in the fibrinogen level and generation of breakdown products in one of the six patients only.

A marked synergism between t-PA and scu-PA and between t-PA and urokinase was recently discovered (Collen et al., unpublished observations, 1986) in rabbits with an experimental jugular vein thrombosis. The simultaneous infusion of the drug combination had an equivalent thrombolytic effect at one-fifth of the dose compared with the separate infusion of either agent. No clear synergism was observed between scu-PA and urokinase. Provided the present observation can be extrapolated to human patients, the combined use of synergic thrombolytic agents may allow not only significant reduction of the total doses, but also

eliminate the systemic fibrinolytic activation and its undesirable breakdown of the hemostatic system.

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